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To isolate new breast tumor suppressor genes GSE library specific for normal breast cells was screened for transforming elements. In parallel, *ING1* candidate tumor suppressor gene, previously isolated through the screening of the same GSE library, was analyzed. *Ing1* was found to be highly evolutionarily conserved gene with complex regulation, which involves generation of alternative transcripts initiated from different promoters and translated into two proteins that differ in size and expression patterns. These proteins have opposite effects on p53-regulated transcription, indicating that *ing1* encodes two products with the properties of a putative tumor suppressor and a putative oncogene. *Ing1* product that works as a p53 antagonist was shown to be highly expressed in different tumor cell lines and primary mouse skin tumors, which might reflect its connection with cancer development. Mice with the heterozygous deletion of *ing1* gene are generated. Analysis of *ing1* knockout animals should give us important insight into its possible role in cancer development.

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Mayuji Yamamura 10/26/1999

PI - Signature Date

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INTRODUCTION

Mutations in tumor suppressor genes play a key role in both genetic predisposition of different forms of cancer and in the etiology of spontaneous malignancies. The goal of our research was the isolation and analysis of tumor suppressor genes whose loss or inactivation is involved in the formation or progression of breast cancer. To do that, we are using genetic suppressor element (GSE) approach based on the expression selection of transforming GSEs, which promote transformation presumably by suppressing tumor suppressor genes from which they derived (1,2). Since we are interested in breast cancer tumor suppressor genes we are using GSE library specific for normal breast cells which is enriched for sequences derived from putative tumor suppressor genes (2). Different selection procedures were applied in order to isolate transforming GSE from the library as a first step towards the isolation of the new breast tumor suppressor genes. In parallel, we are analyzing the *ING1* gene, previously isolated through the screening of the same GSE library. The accumulated observations indicating *ING1* participation in the negative regulation of cell proliferation, control of cellular aging and apoptosis defined *ING1* as a candidate tumor suppressor gene (2,3,4). *ING1* was mapped to human chromosome 13q34 (5), and loss of heterozygosity in this chromosomal locus has been reported in squamous cell carcinomas of head and neck. It was also shown that $p33^{ING1}$ is a component of the p53 signaling pathway, and that $p33^{ING1}$ cooperates with p53 in negative regulation of cell proliferation by modulating p53-dependent transcriptional activation (6). All this characteristics are making *ING1* an extremely interesting subject for analysis.

BODY

In order to isolate GSEs for the breast tumor suppressor gene, I used GSE library prepared from cDNA specific for normal human breast epithelial cells (2). This library, cloned into pLNCX retroviral vector, is enriched for the sequences derived from tumor suppressor genes. In the selection procedures I used MCF10A human pseudonormal immortalized cell line (7) which is non-tumorigenic for nude mice and has low efficiency of growth in semi-solid media as well as low background in spontaneous focus formation. Library was delivered to test cells using BING packaging cell line (8) for virus preparation. Infected cells were used in three different selection procedures: selection for tumorigenicity in nude mice, selection for foci formation, and selection for colony formation in semi-solid agar.

In previous two years library was delivered several times independently into the test cells. Each time part of the cells was used for the detection of infection efficiency and it was shown to be close to 100% in all cases. The rest of infected cells were divided in three parts and each part was used for one of the three selection protocols.

After the subcutaneous injection of infected cells in athymic nude mice none of the animals developed tumors. This selection procedure was repeated several times using independently infected MCF10A cells, and it always gave the same negative result.

Parts of the infected cells were also used in the focus forming assay and semi-solid agar assay. For the focus forming assay cells were plated on 5% serum and incubated for 3-5 weeks. This selection procedure was repeated several times, each time giving significant number of foci. Foci were picked up from the plate and propagated in separate cultures. For the semi-solid agar assay infected cells were plated on soft agar plates and kept for 4-6 weeks. This selection procedure was also repeated several times, each time giving significant number of colonies. All colonies were picked up and propagated in separate cultures. In both assays virus was rescued by cell fusion with packaging cells and used in the second round of selection. After the second round of selection, performed in the same way as the first round, number of transforming elements decreased to only few. GSEs responsible for the transformation were recovered by PCR amplification and recloned into the same retroviral vector. These GSEs were used in the third round of selection, but none of the recloned GSEs was shown to have transforming effects.

In parallel with the selection procedures I also studied the *ING1* gene, previously isolated through the similar screening of the same GSE library. Results obtained in the previous two years were extremely interesting. They are strongly indicating the involvement of *ING1* in cancer formation and development, and since the selection of the GSE library for the transforming elements was mostly giving negative results, I dedicated most of my time in the previous year to study this important gene.

In the first year I isolated mouse homologue of *ING1* and analyzed its structure and expression. *Ing1* was found to be highly evolutionarily conserved gene with complex regulation. Mouse *ing1* is transcribed from three differently regulated promoters. The resulting transcripts share a long common region encoded by a common exon and differ in their 5'-exon sequences encoded by alternative 5'-exons. Two transcripts are translated into a 31kD protein, $p31^{ing1}$, starting from the initiator codon inside the common region. The third transcript encodes a longer protein, $p37^{ing1}$, initiated in 5'-exon and translated in

frame with $p31^{ing1}$. To see details of this work I attached paper accepted for publication in Journal of Biological Chemistry (see Appendix).

In the last year most of the experiments concerning *ing1* were done to address the questions of its function. All previous functional analysis of *ING1* was done using human cDNA expressing the shorter protein, $p33^{ING1}$ (2,3,4,6). As a result, $p33^{ING1}$ was defined as a negative growth regulator that cooperates with p53 in transcriptional activation of p53-responsive genes. Analysis of $p31^{ing1}$, mouse equivalent of $p33^{ING1}$, showed that it has similar biological effects as its human homologue. However, $p37^{ing1}$ was shown to have opposite effects on p53-regulated transcription. Overexpression of $p37^{ing1}$ interferes with the accumulation of p53 protein after DNA damage induced by UV irradiation and attenuates the activation of p53-responsive promoters (for details see Appendix). This means that the two proteins encoded by the *ing1* gene have opposite effects on p53 function. Thus, a single *ing1* gene appeared to simultaneously encode a candidate tumor suppressor gene (p53 cooperator) and a putative oncogene (p53 inhibitor). This means that a simplified view of the potential role of *ING1* in cancer should be revised. *ING1* could be an example of a new genetic mechanism of cancer predisposition involving a balance between the two products of one gene.

It was previously shown that $p33^{ING1}$ can be detected in cells in a complex with p53 by co-immunoprecipitation (6). Since at that time it was not known that *ING1* encodes two different proteins, it was unclear which of the two products of *ING1* was detected in these experiments. New experiments were performed and showed that although $p37^{ING1}$ is co-immunoprecipitated with p53, the shorter *ING1* product is not detectable in the p53 precipitate (for details see Appendix). This observation suggests that differences in the effect of the two *ING1* products on p53 function could be a reflection of differences in their interaction with p53 in the cell.

An important observation that might reflect the connection between the long protein product of *ING1* and cancer development was made when *ING1* mRNA expression was analyzed in a large number of primary mouse skin tumors induced by a classical two-step carcinogenesis (DMBA-TPA treatment). High levels of mRNA for the longer product of the *ing1* gene (p53 antagonist) were found, with one exception, in all RNA tumor samples isolated from 8 papillomas and 12 carcinomas in comparison with normal or hyperplastic TPA-treated skin. The expression of mRNAs encoding the shorter *ing1* product (p53 cooperator) was not elevated in the tumors. It is noteworthy that DMBA-induced skin tumors in the vast majority of cases retain wild type p53. Consistently, western analysis of *ING1* protein products in a number of tumor cell lines revealed that the majority of them, unlike normal cells, expressed, almost exclusively, the longer variant of the *ING1* protein.

To understand *ING1* role in tumor formation we are planning to analyze the consequences of experimental deregulation of *ING1* expression in tumor cell lines, to analyze relative expression of two *ING1* products in tumor cells and to analyze expression of $p37^{ING1}$ by immuno-histochemical staining of histological sections of tumor samples. To analyze expression levels of *ING1* in different human tumors we depend on the availability of antibodies to different variants of the *ING1*-encoded proteins. It is impossible to generate antibodies that would specifically recognize only the short *ING1* product $p31/p33^{ING1}$ since this protein is the truncated version of the longer one, $p37^{ING1}$. Monoclonal and polyclonal antibodies, recognizing all forms of the *ING1* proteins, were available and were used in previous studies. They can be used for the detection of relative expression of $p37^{ING1}$ and $p31/p33^{ING1}$ on western blots but cannot distinguish between these products in immunohistochemical staining. In order to selectively detect $p37^{ING1}$, we generated a panel of monoclonal antibodies against this protein synthesized and

purified from E.coli. Their specificity and applicability to western blots and immunohistochemical staining was determined using cells overexpressing different *ING1* cDNAs and three hybridoma clones were chosen for future applications.

In order to make *ing1* deficient animals we decided to delete common exon of *ing1* in their genome. To do that, we applied widely used technique of positive/negative selection (9). In positive/negative selection, the targeting vector contains a neomycin resistance (neo) gene within homologous regions of the target sequence and a herpes simplex virus thymidine kinase (*TK*) gene outside of these regions. The target construct carries two regions approximately 4 kb each in the same orientation on both sides of the major exon of the *ing1* gene. The resulting recombinant allele is expected to have all the common exon sequences of *ing1* deleted leading to complete inactivation of the gene. ES cells were transfected with the construct and selected in both G418 and ganciclovir. The selected cells were used for creation of chimeric animals. To facilitate analysis of chimeras, donor ES cells (homozygous agouti 129/Sv mice) and recipient host blastocytes (homozygous for black C57BL/6 mice) are derived from strains of mice that differ in coat color. We have already obtained several chimeric mice. Males with a large contribution of agouti ES cells were crossed with C57BL/6 females. Among the offspring of this crossing we have several agouti animals with the heterozygous deletion of the common exon of *ing1* gene. Homozygous knockout mice will be obtained from intercross breeding of heterozygous animals. This work is in progress.

APPENDIX

1. Key research accomplishments:

   - It was found that *ing1* gene was transcribed from three differently regulated promoters; alternative transcripts are translated into two proteins different in size and expression patterns.
   - Two *ing1* protein products have opposite effects on p53-regulated transcription, indicating that *ing1* encodes two products with the properties of a putative tumor suppressor and a putative oncogene.
   - It was shown that the longer *ing1* protein product has high expression in different tumor cell lines and primary mouse skin tumors, which is indicating its involvement in tumor formation and development.
   - Monoclonal antibodies specifically recognizing only the longer *ING1* protein product were made.
   - Mice with the heterozygous deletion of the *ing1* common exon are generated.

2. Reportable outcomes:

- Conference presentation:

  Zeremski, M., and Gudkov, A.V. Structure and expression of the mouse homologue of *ING1* candidate tumor suppressor gene. Eighty-ninth annual meeting of American Association for Cancer Research. March 28 - April 1, 1998, New Orleans, LA.

- Publication (attached):

  Zeremski, M., Hill, J.E., Kwek, S.S., Grigorian, I.A., Gurova, K.V., Garkavtsev, I.V., Diatchenko, L., Koonin E.V., and Gudkov, A.V. (1999). Structure and regulation of the mouse *ing1* gene: three alternative transcripts encode two PHD finger proteins that have opposite effects on p53 function. J. Biol. Chem. (in press)

- U.S. Provisional Patent Application No. 60/118,941 filed on February 4, 1999:
  $P37^{ING1}$ compositions and methods
  Inventors: Gudkov, A., Zeremski, M., Gurova, K.V., Grigorian, I.A.

- Principal investigator, Marija Zeremski, obtained Ph.D. degree based on work supported by this award

- GenBank™ accession numbers for the sequences isolated in the work supported by this award are AF177753-AF177757

- Based on work supported by this award grant application was submitted to NIH. Grant application No. R01CA60730.

REFERENCES

1. Ossovskaya, V.S., Mazo, I.A., Chernov, M.V., Chernova, O.B., Strezoska, Z., Kondratov, R., Stark, G.R., Chumakov, P.M., and Gudkov, A.V. (1996). Dissection of p53 functions by genetic suppressor elements: distinct biological effects of separate p53 domains. Proc. Natl. Acad. Sci. USA 93, 10309- 10314.
2. Garkavtsev, I.A., Kazarov, A.R., Gudkov, A.V., and Riabowol, K. (1996). Suppresion of the novel growth inhibitor $p33^{ING1}$ promotes neoplastic transformation. Nature Genetics 14, 415-420.
3. Garkavtsev, I.A., and Riabowol, K. (1997). Extension of the replicative life span of human diploid fibroblasts by inhibition of the $p33^{ING1}$ candidate tumor suppressor. Mol. Cel. Biol. 17, 2014-2019.
4. Helbing, C.C., Veillette, C., Riabowol, K., Johnston, R.N., and Garkavtsev, I. (1997). A novel candidate tumor suppressor, *ING1*, is involved in the regulation of apoptosis. Cancer Res. 57, 1255-1258.
5. Zeremski, M., Horrigan, S.K., Grigorian, I.A., Westbrook, C.A., and Gudkov, A.V. (1997). Localization of the candidate tumor suppressor gene *ING1* to human chromosome 13q34. Somat. Cell Mol. Genet. 23, 233-236.
6. Garkavtsev, I., Grigorian, I.A., Ossovskaya, V.S., Chernov, M.V., Chumakov, P.M., and Gudkov, A.V. (1998). The candidate tumor suppressor $p33^{ING1}$ cooperates with p53 in cell growth control. Nature 391, 295-298.
7. MCF10A cell line: ATCC Cell Lines and Hybridomas eight edition 1994. Under CRL-10317.
8. Pear, W.S., Nolan, G.P., Scott, M.L., and Baltimore, D. (1993). Production of high titer helper-free retroviruses by transient transfection, Proc. Natl. Acad. Sci. USA 90, 8392-8396.
9. Mansour, S.L., Thomas, K.R., and Capecchi, M.R., (1988). Disruption of the protooncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. Nature 336, 348-352.

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Structure and Regulation of the Mouse *ing1* Gene

THREE ALTERNATIVE TRANSCRIPTS ENCODE TWO PHD FINGER PROTEINS THAT HAVE OPPOSITE EFFECTS ON p53 FUNCTION*

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The human *ING1* gene encodes nuclear protein p33^{ING1}, previously shown to cooperate with p53 in cell growth control (Garkavtsev, I., Grigorian, I. A., Ossovskaya, V. S., Chernov, M. V., Chumakov, P. M., and Gudkov, A. V. (1998) *Nature* 391, 295–298). p33^{ING1} belongs to a small family of proteins from human, mouse, and yeast of approximately the same size that show significant similarity to one another within the C-terminal PHD finger domain and also contain an additional N-terminal region with subtle but reliably detectable sequence conservation. Mouse *ing1* is transcribed from three differently regulated promoters localized within a 4-kilobase pair region of genomic DNA. The resulting transcripts share a long common region encoded by a common exon and differ in their 5′-exon sequences. Two transcripts are translated into the same protein of 185 amino acids, the mouse equivalent of the human p33^{ING1}, while the third transcript encodes a longer protein that has 94 additional N-terminal amino acids. Overexpression of the longer protein interferes with the accumulation of p53 protein and activation of p53-responsive promoters after DNA damage. Between the two products of *ing1*, only the longer one forms a complex with p53 detectable by immunoprecipitation. These results indicate that a single gene, *ing1*, encodes both p53-suppressing and p53-activating proteins that are regulated by alternative promoters.

The *ING1* gene was identified as a result of a functional screening of genes, the suppression of which is associated with neoplastic transformation (1). Inhibition of *ING1* expression by antisense RNA promotes anchorage-independent growth in mouse breast epithelial cells, increases the frequency of focus formation in NIH 3T3 cells, and prolongs the life span of diploid human fibroblasts in culture. *ING1* expression is up-regulated in senescent human fibroblasts (2), and ectopic expression of *ING1* cDNA leads to G_1 arrest or promotes apoptosis in several cell types (3). The accumulated observations indicating *ING1* participation in the negative regulation of cell proliferation, control of cellular aging, and apoptosis have defined *ING1* as a candidate tumor suppressor gene.

We have recently found that the biological effects of *ING1* and *p53* are interrelated and require the activity of both genes. Neither of the two genes can, on its own, cause growth inhibition when the other one is suppressed (4). Furthermore, activation of transcription from the *p21/WAF1* promoter, a key mechanism of p53-mediated growth control, depends on the expression of *ING1*. A physical association between p33^{ING1} and p53 proteins is detected by immunoprecipitation. These results indicated that p33^{ING1} is a component of the p53 signaling pathway and that p33^{ING1} cooperates with p53 in negative regulation of cell proliferation by modulating p53-dependent transcriptional activation.

Despite the apparent importance of *ING1* in the control of cell proliferation, our knowledge of expression, regulation, and function of this gene remains incomplete. Moreover, GenBank™ contains two *ING1* mRNA sequences differing in their 5′-ends. The origin of these differences is unknown and requires explanation. So far, all of the information about the function of p33^{ING1} was obtained from *in vitro* experiments that involved ectopic expression of *ING1* cDNA or its suppression by antisense RNA. The analysis of the structure of the *ING1* gene and its regulation *in vivo* are essential steps toward the understanding of its function and involvement in developmental and physiological processes. This is particularly important due to the cooperation between *ING1* and *p53*, which suggests that the functioning of the p53 signaling pathway could be dependent on the regulation of *ING1* expression.

In the present work, we used the mouse ortholog of the human *ING1* gene for detailed structural and expression studies, with the goal of subsequently utilizing it as a system for extensive genetic analysis. *ing1* was found to be a highly evolutionarily conserved gene with complex regulation, which involves generation of alternative transcripts initiated from different promoters and translated into proteins that differ in structure and expression patterns. Moreover, these proteins have opposite effects on p53-regulated transcription, indicating that *ing1* encodes two products with the properties of a putative tumor suppressor and a putative oncogene.

MATERIALS AND METHODS

Plasmids and Libraries—Retroviral vector pLXSN, used for the introduction of mouse and human *ING1* cDNA in NMuMG, 10(1), and ConA cells, was provided by A. Dusty Miller (5). Retroviral vector pLXIG was constructed on the basis of pLXSN vector by substituting SV40 promoter and *neo* sequences with the internal ribosome entry site of encephalomyocarditis virus (6) followed by the enhanced green fluorescent protein sequence (7). This vector permits us to translate both the gene of interest (cloned upstream of the internal ribosome entry

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site) and enhanced green fluorescent protein from a single bicistronic mRNA transcribed from Moloney murine leukemia virus long terminal repeat promoter. A cDNA library from senescent mouse embryonic fibroblasts was constructed using SuperScript system (Life Technologies, Inc.) according to the manufacturer's protocol and cloned into the λ phage λ gt22A vector. Stratagene's 129SVJ mouse genomic library cloned into the λ FIX II vector was used for the isolation of the mouse genomic, *ing1*-containing clones.

Cell Lines—Cultures of mouse embryonic fibroblasts were obtained from 10-day-old embryos. 10(1) cell line, a derivative of Balb/c 3T3 cells that spontaneously deleted both p53 alleles (8), was kindly provided by Arnold Levine. Pseudonormal mouse mammary gland epithelial cell line (NMuMG) was obtained from the ATCC collection. Ecotropic retroviral packaging cell line BOSC23 (9) was kindly provided by Warren Pear and David Baltimore (Massachusetts Institute of Technology). The ConA cell line, a derivative of Balb/c 3T3 cells 12-1 (8) with wild type p53, was described earlier (10). It carries the *lacZ* gene encoding β-galactosidase *Escherichia coli* under the control of the p53-dependent promoter and therefore allows monitoring p53 transcriptional activation by a routine X-gal staining. All cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum supplemented with penicillin and streptomycin. For the serum starvation experiments, 10(1) and NMuMG cells were kept with 0.5% fetal bovine serum for 48 h (NMuMG cells) or 36 h (10(1) cells). For the contact inhibition experiments, cells cultures were used 48 h (NMuMG) or 36 h (10(1)) after they became confluent. NMuMG and 10(1) cells were irradiated with 10 grays of γ-radiation and used 24 h after treatment. Mouse embryo fibroblasts were propagated until they reached senescence. Populations of ConA cells, expressing different levels of $p37^{ING1}$, were generated by transduction with pLXIG vector, carrying $p37^{ING1}$ cDNA, followed by fluorescence-activated cell sorting of cells with different levels of fluorescence.

Animals—Organs and embryos of FVB/N mice were used for the RNA isolation and preparation of whole body sections of embryos using a cryostatic microtome for histoblot hybridization.

Hybridization Screening of cDNA and Genomic Libraries—cDNA and genomic library screenings were done according to the standard protocols (11) using ^{32}P-labeled human and mouse *ing1* cDNA probes, respectively.

Fn1

5′- and 3′-RACE[1]—Alternative 5′-ends of the mouse *ing1* were isolated from mouse spleen and mouse brain cDNAs using the Marathon-ready cDNA kit (CLONTECH), according to the protocol suggested by the manufacturer. AP1 adaptor-specific sense primer, provided by CLONTECH, and the *ing1*-specific antisense primer (5′-CCATCTGACTCACGATCTGGATCTTC-3′) were used for PCR. Nested PCR was performed using AP2 adaptor-specific sense primer, provided by CLONTECH, and *ing1*-specific antisense primer (5′-CTGCGGATCAGGGCCCTCTGGATGC-3′). Precise determination of the 5′- and 3′-ends of the mouse *ing1* transcripts was done using the Marathon-2 cDNA amplification Kit based on the new SMART PCR cDNA synthesis technology (CLONTECH). It is based on the recently identified ability of Moloney murine leukemia virus reverse transcriptase to add several nucleotides to the 3′ terminus of first-strand cDNA during the reverse transcriptase reaction (12). Briefly, when reverse transcriptase reaches the 5′-end of the mRNA, it switches templates and continues synthesizing the SMART template-switching oligonucleotide. The resulting single-stranded cDNA contains the complete 5′-end of the mRNA as well as the sequence complementary to the template-switching oligonucleotide and is then selectively amplified by PCR. In these experiments, poly(A) RNA preparations isolated from the thymus and testis were used. The following sequences were used for the synthesis of antisense *ing1*-specific primers: 5′-AGGTGTGGTGGGATCGGCAACGC-3′ (for isoform 1a), 5′-CGCGGGGAGCCAGAGCAGAGAAGGT-3′ (isoform 1c), and 5′-GGCGTGGCCTGTCATTGTCGCTG-3′ (isoform 1b). *ing1*-specific sense primer 5′-GCGTGCTTCTTGCTACCAT-3′ was used for the PCR amplification of the 3′-end.

Sequence Analysis—Sequencing was done using a Sequenase version 2.0 DNA sequencing kit (U.S. Biochemical Corp.) or done by the University of Chicago Cancer Research Center DNA Sequencing Facility. In all cases, both strands were read using multiple vector-specific or gene-specific primers. Protein sequence data base searches were performed using the gapped BLASTP program and the PSI-BLAST program that iterates the search using profiles constructed from BLAST hits as queries for subsequent iterations (13). Multiple sequence alignments were constructed using the Gibbs sampling option of the MACAW program (14, 15). GenBank™ accession numbers for the described sequences are AF177753–AF177757.

Southern, Northern, and Western analyses were done according to standard protocols. IgG1 mouse monoclonal antibody against human recombinant $p33^{ING1}$ (16) was used for the detection of *ing1*-encoded proteins. Anti-p53 monoclonal mouse IgG antibody Ab-1 was obtained from Calbiochem. p21 rabbit polyclonal IgG antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Immunoprecopitation Analysis—Immunoprecipitation of p53 was carried out using 1 μg of DO-1 (Santa Cruz Biotechnology) and 1 μg of Ab-1 (Calbiochem) antibodies per 0.5 ml of cell lysate. Cell lysate was obtained by transient transfection of the corresponding vectors by LipofectAMINE Plus (Life Technologies, Inc.) into p53-negative Saos-2 cells. Each transfected plate of cells was lysed in 0.5 ml of modified radioimmune precipitation buffer (25 mM Tris, pH 7.4, 125 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate with protease inhibitor). Sepharose-protein A (Amersham Pharmacia Biotech) was used to pull out the antibody-protein complexes, and pellets were washed four times with modified radioimmune precipitation buffer with 0.5% Nonidet P-40. Pellets were boiled in SDS-loading dye and run on 10% SDS-polyacrylamide gel electrophoresis. Western blot was carried out with anti-HA-biotin antibodies, with clone 12CAS (Roche Molecular Biochemicals), or with anti-p53 biotinylated antibodies (Roche Molecular Biochemicals) according to standard protocols.

In Situ Hybridization on Nitrocellulose ("Histoblotting")—Whole body sections, 20 μm thick, were prepared from frozen embryos embedded in blocks of Tissue-Tek O.C.T. and stored at −70 °C. Sections were placed on nitrocellulose (Schleicher & Schuell) to prepare histoblots as described previously (17). Histoblots were hybridized with ^{33}P-labeled RNA probes synthesized using Ambion's MAXIscript *in vitro* transcription kit. *ing1*-specific sense and antisense probes were synthesized on the pBLUESCRIPT plasmid with the fragment of *ing1* cDNA corresponding to the PHD finger domain using T3 polymerase for the antisense and T7 polymerase for the sense probe. β-Actin antisense RNA was synthesized using the template provided by Ambion. Histoblots were incubated in prehybridization solution (0.75 mg/ml yeast tRNA, 0.75 mg/ml poly(A), 50% formamide, 0.3 M Tris, pH 8.0, 1 mM EDTA, 5× Denhardt's solution, 10% dextran sulfate, 10 mM dithiothreitol) at 42 °C for 1–4 h. Hybridization was carried out for 12–24 h at 42 °C in the same solution containing 1–5 × 10^7 cpm of probe/ml. After hybridization, histoblots were washed in 4× SSC at room temperature for 20 min, in 2× SSC, 0.04 μg/ml RNase A at 37 °C for 30 min, in 2× SSC at 37 °C for 30 min, in 1× SSC at 60 °C for 15–30 min, and finally in 0.1× SSC at 60 °C for 15–30 min and exposed to x-ray film for 2–7 days.

RESULTS

Multiple Transcripts of the Mouse ing1 Gene Differ in Their 5′-End Sequences—In order to isolate the mouse ortholog of the *ING1* gene, we screened a cDNA library prepared from senescent mouse embryonic fibroblasts, using human *ING1* as a probe. The choice of the library was determined by the fact that *ING1* is expressed at higher levels in senescent, compared with normal, human fibroblasts (2). As a result, several clones were isolated, and the two longest were sequenced. The clones were identical to each other and highly similar to human *ING1* through most of their length, except for the 5′-ends, which were different and not homologous to the human gene (Fig. 1*A*). This observation could be an indication of alternative splicing of mouse *ing1*; however, it could also be potentially explained by cloning artifacts that occurred during the cDNA library preparation. To determine the structure of 5′-end sequences of *ing1*, we used a 5′-RACE technique for the isolation of cDNA sequences corresponding to the 5′-termini of *ing1* mRNA. cDNA was synthesized from mouse spleen and brain mRNA and ligated to synthetic adaptors. The cDNA was amplified by PCR using a sense primer specific for the adaptor and an antisense primer specific for the common part of *ing1* located close to the divergent region. Two fragments of different size were subsequently cloned, sequenced, and compared with the clones isolated from the cDNA library. The results of this comparison are schematically presented in Fig. 1*A*. One of the 5′-RACE prod-

F1

[1] The abbreviations used are: RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

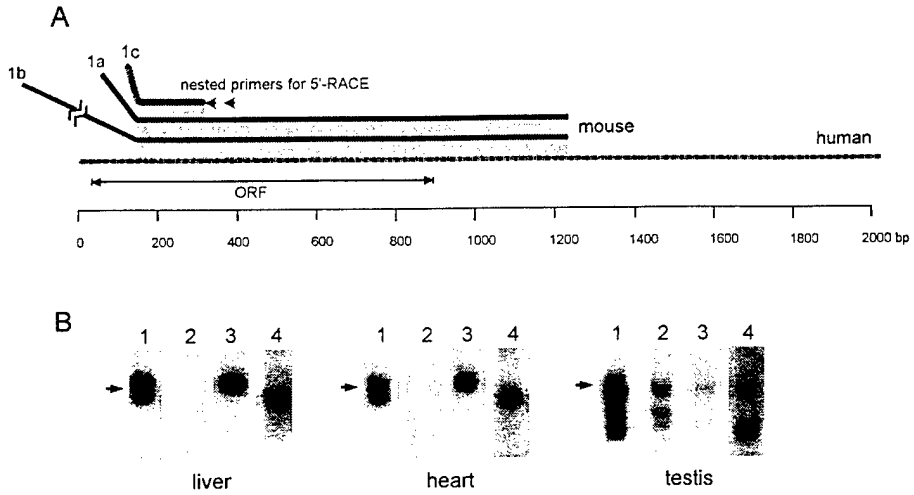


FIG. 1. **Cloning of different variants of mouse *ing1* cDNA and identification of their corresponding mRNA transcripts.** *A*, schematic alignment of the mouse *ing1* cDNA clones and 5′-RACE products with human *ING1* cDNA. 1a and 1b represent mouse *ing1* cDNAs isolated from the library of senescent mouse embryonic fibroblasts. A 5′-RACE reaction gave two products, one identical to 1b and another shown as 1c. The position of the gene-specific PCR primers used for the RACE reaction is indicated. Mouse *ing1* sequences are aligned with human *ING1* cDNA. The open reading frame for the human *ING1* clone is indicated. *B*, analysis of *ing1* transcription by Northern hybridization. *ing1* expression in mouse liver, heart, and testis was analyzed using a multiple-tissue Northern blot (CLONTECH Laboratories), which was hybridized with the probe corresponding to the common part of *ing1* (*lane 1*), the 5′-end of isoform 1a (*lane 2*), the 5′-end of isoform 1b (*lane 3*), or the 5′-end of isoform 1c (*lane 4*). Probes were obtained by PCR using *ing1*-specific primers. An *arrow* shows the position of the RNA marker, 2.37 kilobases in size.

ucts was identical to one of the previously isolated cDNA clones. Another product revealed the third variant of *ing1* cDNA, which again consisted of common and unique regions, with the junction located at exactly the same position as in the other sequences. Fig. 1*A* shows schematic alignment of the isolated mouse *ing1* cDNA clones and the 5′-RACE products. All variants are identical to each other (and homologous to human *ING1*) up to the same nucleotide and differ only in their 5′-ends.

In order to verify that the observed cDNA structure reflects naturally existing transcripts, we analyzed *ing1* mRNA species by Northern hybridization using probes that represent common or specific regions of the isolated cDNAs. As shown in Fig. 1*B*, the probe for the common region revealed multiple transcripts in RNA isolated from mouse liver, heart, and testis. Probes specific for individual transcripts, however, showed more simple hybridization patterns, which in combination covered the whole set of transcripts found by hybridization with the common probe. These results indicated that the cloned sequences represent the majority of multiple transcripts of *ing1* synthesized in the thymus and spleen.

Mapping of Coding Regions of ing1 in Mouse Genomic DNA—Comparison of sequences of individual cDNA clones showed differences in their 5′ regions, suggesting that *ing1* has multiple alternative 5′-exons (Fig. 1). To verify this hypothesis, we determined the structure of the mouse *ing1* gene. We isolated phage clones carrying sequences homologous to the mouse *ing1* cDNA by hybridization screening of a mouse genomic library. These clones were mapped by restriction digestion analysis, in combination with Southern blot hybridization, with the probes corresponding to the different *ing1* parts. The interpretation of the results obtained is shown in Fig. 2*A*. F2

Comparison of genomic and cDNA sequences of *ing1* using PCR with different *ing1*-specific primers (data not shown) as well as with the sequencing data showed that most of the transcribed sequences of the *ing1* gene come from a single exon. Alternative 5′-ends are encoded by different exons positioned upstream from the common exon. Comparison of the sequences of cDNA and genomic clones, as well as 5′-RACE products, revealed three isoforms of mouse *ing1* that differ from each other only in their 5′-ends, which also indicates that each isoform is most probably expressed from its own promoter. Southern blot hybridization analysis of *ing1*-related sequences in the mouse genome indicated that *ing1* is a single gene with no obvious close family members (data not shown).

The length of the isolated cDNA clones appeared to be significantly shorter than that of the mRNA species detected by Northern hybridization (Fig. 1*B*), suggesting that part of the transcribed sequences were missing from the isolated cDNAs. To determine the exact start sites of *ing1* transcription, we used a new procedure called "SMART-based 5′-RACE" as described under "Materials and Methods." Using this method, we were able to extend the cDNA sequences for the 5′-end of isoform 1b. This exon contains an extremely G/C-rich region that blocked DNA elongation during the original 5′-RACE reaction (Figs. 2*B* and 3) and complicated sequencing of the final, long 5′-RACE product. Therefore, the transcription start site of isoform 1b was not precisely identified, since it was estimated based on the size of the 5′-RACE product and the analysis of the genomic sequence.

Comparing the 3′-ends of human and mouse *ING1* cDNAs showed that although the mouse transcript was flanked by a poly(A) stretch, its 3′-untranslated region was significantly shorter than the human one. Analysis of the genomic clone with the mouse *ing1* sequence revealed the presence of a long poly(A) stretch that could potentially be used as a primer-binding sequence for reverse transcription initiated from oligo(dT) primers (Fig. 3*C*). Moreover, the alignment with the 3′-end of the human *ING1* cDNA sequence resumes downstream from this genomic poly(A) stretch. The exact end of *ing1* transcription in mice was determined using the same method applied for the generation of 5′-sequences. Mouse *ing1* cDNA was, in fact, found to be longer than was originally thought (Figs. 2*B* and 3). Sequence comparison showed that mouse and human *ING1* transcripts terminate at the same point and share significant levels of similarity up to the very 3′-ends. F3

Sequence Analysis of ing1—Nucleotide and predicted amino acid sequences of the three mouse *ing1* cDNA isoforms and the promoter regions for all three alternative transcripts are shown in Fig. 3. It was found that *ing1* isoforms 1a and 1c have several tightly clustered transcription start sites. Sequences upstream of the initiation sites (putative promoters) lack TATA boxes,

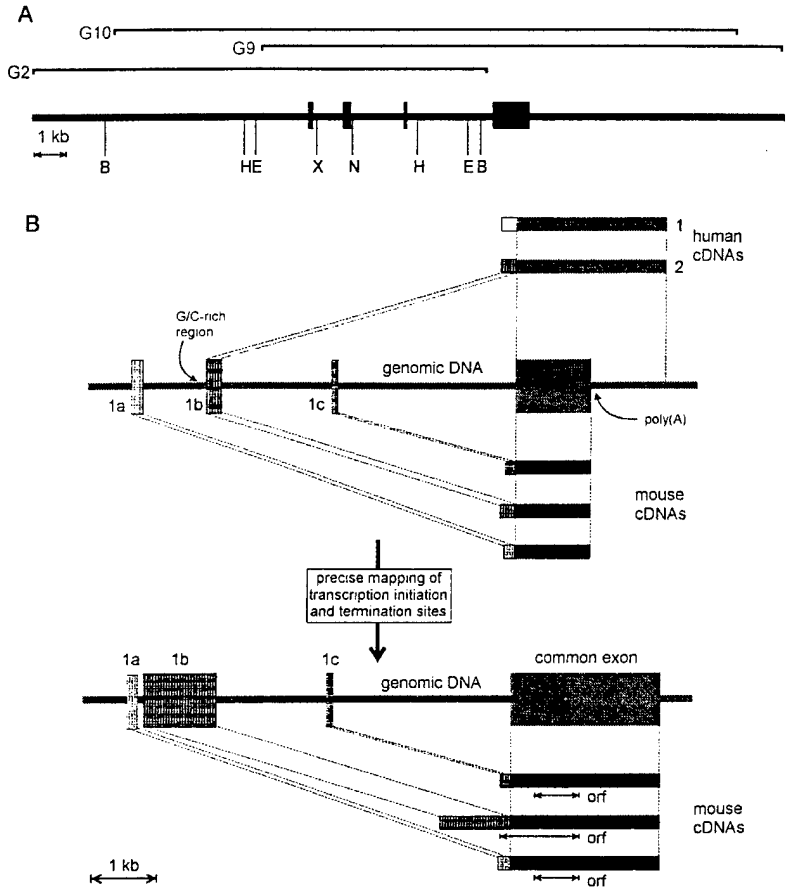


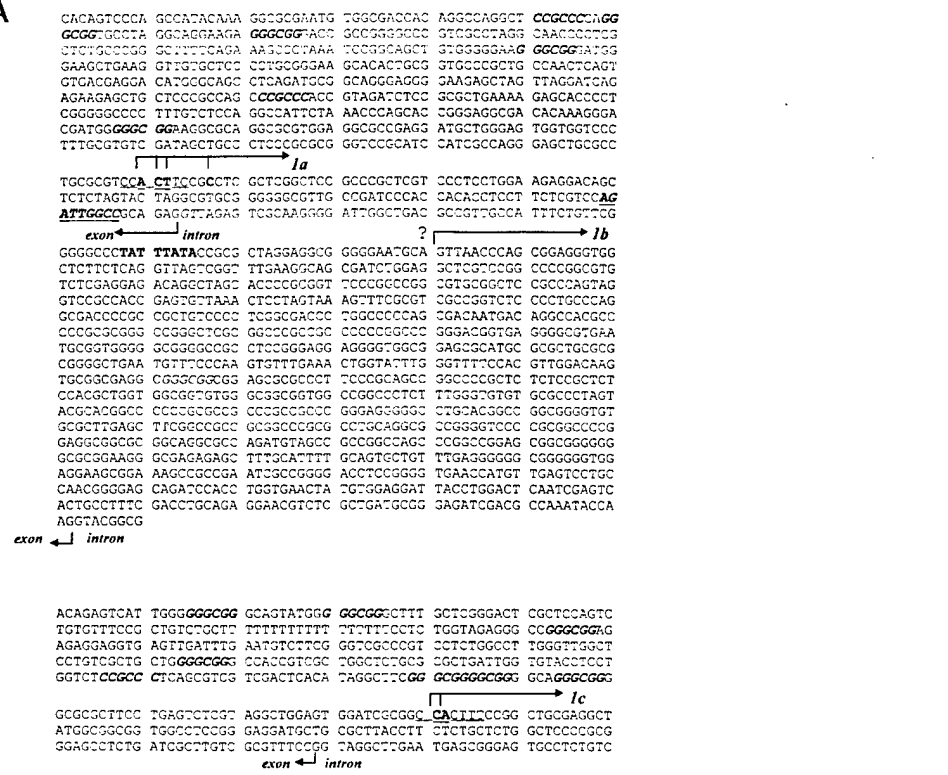
FIG. 2. **Mapping of mRNA-coding regions within the mouse *ing1* locus.** *A*, map of the mouse genomic region containing *ing1* cDNA-related sequences. Three *ing1*-positive genomic clones were isolated from the mouse genomic library using *ing1* cDNA as a probe (G2, G9, and G10), and analyzed by restriction digestion and Southern blot hybridization. This analysis, in combination with the PCR data and with partial sequencing of the genomic clones, allowed us to determine the positions of the *ing1* exons. *B*, determination of transcription initiation and termination sites in the *ing1* gene. The *upper panel* shows the genomic map with the positions of the *ing1* exons and the structure of *ing1* cDNA clones as determined after cDNA library screening and 5′-RACE analysis. The structure of two known human *ING1* cDNA isoforms in relation to mouse *ing1* sequences is shown *above* the genomic DNA. The *lower panel* demonstrates the final structure of the *ing1* gene determined as a result of precise mapping of transcription initiation and termination sites. In the *upper panel*, the position of the G/C-rich region in the 5′-end of clone 1b and the poly(A) region in the 3′ part of the *ing1* transcripts are indicated, which interfered with the polymerase chain reaction during the original 5′-RACE.

but they do contain a sequence corresponding to the loose initiator consensus PyPyAN(T/A)PyPy that includes the transcription start sites (Fig. 3). In both cases, areas upstream from the initiator are extremely GC-rich with multiple Sp1-binding sites. All of these features are typical of TATA-less promoters (18, 19). Binding sites of inducible factors that are usually present further upstream in the promoter area (*e.g.* CAAT box, Oct-1 and Oct-2 binding site, NF-κB, or ATF binding site) were not found in these promoters. Transcription of *ing1* isoform 1b is estimated to start approximately 30 nucleotides downstream from the TATA-like box. A putative CAAT box is present 60 nucleotides upstream from the TATA-like sequence. Since the transcription initiation sites for isoforms 1a and 1b are only about 200 base pairs apart, there is a possibility that their promoters share some of the regulatory sequences including, for example, a number of Sp1 binding sites located upstream of this area.

All three alternative transcripts of mouse *ing1* contain the same long open reading frame, although the sizes of the predicted proteins are different. While isoform 1b encodes a protein of 279 amino acids, the other two isoforms are predicted to encode a shorter protein product of 185 amino acids, which lacks 94 N-terminal amino acids. Translation of these two products is expected to start from the initiation codon, which is located at the beginning of the common exon. Alignment of the predicted amino acid sequences with that of human $p33^{ING1}$ (GenBank™ accession number AF044076) revealed high similarity between the mouse and human proteins (89% sequence identity) (Fig. 4). F4

The nonredundant protein sequence data base at NCBI was searched using the *ing1*-encoded protein sequence as a query, and a highly significant sequence similarity was detected with a human paralog of $p33^{ING1}$. These included three uncharacterized proteins from the budding yeast *Saccharomyces cerevisiae* and their homologue from fission yeast *Schizosaccharomyces pombe* (probability of the similarity being observed by chance $<10^{-12}$). All of these proteins are approximately the same size and contain a C-terminal PHD finger domain (20, 21). The sequence conservation in the PHD domain in these six protein sequences is striking. There are 13 invariant residues in addition to the eight metal-chelating cysteines and histidines that are conserved in all PHD fingers (Fig. 4*B*). Multiple alignment analysis resulted in the delineation of an additional N-terminal region that is conserved in these proteins (Fig. 4*B*). This region consists of approximately 100 amino acid residues, includes two distinct conserved motifs, and shows a fairly subtle similarity that was not statistically significant in the context of the screening of the complete data base, except for the conservation between the two human paralogs. However, in the reduced search space defined by the presence of the PHD finger, it was shown that the probability of finding this level of similarity by chance was less than 10^{-13} for the distal motif and 10^{-5} for the proximal motif.

Expression of ing1 Transcripts in Adult and Embryonic Mouse Tissues—We analyzed the expression of *ing1* mRNA in the organs of adult mice and mouse embryos at different stages of development by Northern blot hybridization. The probe representing the common exon of the *ing1* gene revealed multiple mRNA size classes that represent alternative transcripts of *ing1* (Fig. 5*A*). Overall *ing1* mRNA expression is most abundant in thymus and testis. Much lower levels were detected in the rest of the tissues tested, and they also differed in the content and relative intensity of the hybridizing bands. The same pattern of *ing1* mRNA expression was observed in p53-deficient mice, which showed no direct effect by p53 on *ing1* regulation (data not shown). In embryos, the highest expres- F5



C

Exon 1a

ACTTCCGCCTCGCTCGGCTCCGCCCGCTCGTCCCTCCTGGAAGAGGACAGCTCTCTAGTA
60
CTAGGCGTGCGGGGGGCGTTGCCGATCCCACCACACCTCCTTCTCGTCCAGATTGGCCGC
120
ACAG
124

Exon 1b

GTTAACCCAGCGGAGGGTGGCTCTTCTCAGGTTAGTCGGTTTGAAGGCAGCGATCTGGAG
60
GCTCGTCCGGCCCCGGCGTGTCTCGAGGAGACAGGCTAGCACCCCGCGGTTCCCGGCCGG
120
CGTGCGGCTCCGCCCAGTAGGTCCGCCACCGAGTGTTAAACTCCTAGTAAAGTTTCGCGT
180
CGCCGGTCTCCCCTGCCCAGGCGACCCCGCCGCTGTCCCCTCGGCGACCCTGGCCCCCAG
240
CGACAATGACAGGCCACGCCCCCGCGCGGGCCGGGCTCGCGGCCCGCCGCCCCCCGGCCC
300
GGGACGGTGAGGGGCGTGAATGCGGTGGGGGCGGGGCCGCCTCCGGGAGGAGGGGTGGCG
360
GAGCGCATGCGCGCTGCGCGCGGGGCTGAATGTTTCCCAAGTGTTTGAAACTGGTATTTG
420
GGTTTTCCACGTTGGACAAGTGCGGCGAGGCGGGCGGCGGAGCGCGCCCTTCCCGCAGCC
480
GGCCCCGCTCTCTCCGCTCTCCACGCTGGTGGCGGTGTGGGCGGCGGTGGCCGGCCCTCT
540
TTGGGTGTGTGCGCCCTAGTACGCACGGCCCCCCGCGCCGCCCGCCGCCCGGGAGGGGGC
600
CTGCACGGCCGGCGGGGTGTGCGCTTGAGCTTCGGCCGCCGCGGCCCGCGCCTGCAGGCG
660
CCGGGGTCCCCGCGGCCCCGGAGGCGGCGCGGCAGGCGCCAGATGTAGCCGCCGGCCAGC
720
CCGGCCGGAGCGGCGGGGGGGCGCGGAAGGGCGAGAGAGCTTTGCATTTTGCAGTGCTGT
780
TTGAGGGGGGCGGGGGGTGGAGGAAGCGGAAAGCCGCCGAATCGCCGGGGACCTCCGGGG
840
TGAACC***ATG***TTGAGTCCTGCCAACGGGGAGCAGATCCACCTGGTGAACTATGTGGAGGAT
900
M L S P A N G E Q I H L V N Y V E D

TACCTGGACTCAATCGAGTCACTGCCTTTCGACCTGCAGAGGAACGTCTCGCTGATGCGG
960
Y L D S I E S L P F D L Q R N V S L M R

GAGATCGACGCCAAATACCAAG
982
E I D A K Y Q

Exon 1c

CACTTTCCGGCTGCGAGGCTATGGCGGCGGTGGCCTCCGGGAGGATGCTGCGCTTACCTT
60
CTCTGCTCTGGCTCCCCGCGGGAGCCTCTGATCGCTTGTCGCGTTTCCG ***109***

Common exon

AGATCCTGAAGGAGCTGGACGACTACTATGAGAAGTTCAAACGGGAGACAGACGGCACCC
60
E I L K E L D D Y Y E K F K R E T D G T

AGAAGCGCCGGGTACTGCACTGCATCCAGAGGGCCCTGATCCGCAGCCAGGAGCTAGGCG
120
Q K R R V L H C I Q R A L I R S Q E L G

ATGAGAAGATCCAGATCGTGAGTCAG***ATG***GTGGAGCTGGTGGAGAACCGCAGCAGACAGG
180
D E K I Q I V S Q M V E L V E N R S R Q

TGGACAGTCACGTGGAGCTCTTCGAAGCACACCAGGACATCAGTGACGGCACTGGTGGCA
240
V D S H V E L F E A H Q D I S D G T G G

GCGGCAAGGCGGGCCAGGACAAGTCGAAGAGTGAGGCCATCACACAGGCAGATAAGCCGA
300
S G K A G Q D K S K S E A I T Q A D K P

ATAACAAGCGGTCCAGGAGGCAGCGAAACAATGAGAATCGAGAGAACGCGTCGAATAATC
360
N N K R S R R Q R N N E N R E N A S N N

FIG. 3. **Sequences of the putative promoter areas of the *ing1* gene, *ing1* cDNAs, and their predicted protein products.** *A*, sequence of the genomic region with the putative promoter areas of cDNA isoforms 1a and 1b; the start sites of transcription of these two variants are marked

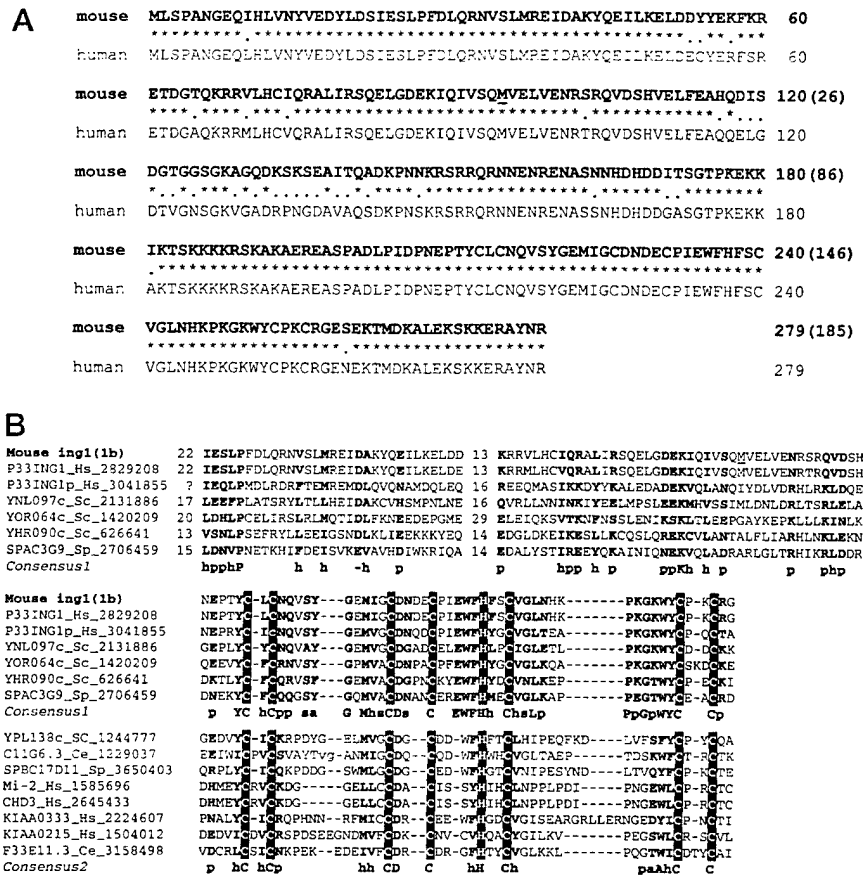


FIG. 4. **Protein sequence analysis of p37^{ing1}.** *A*, alignment of amino acid sequences of mouse *ing1* 1b isoform and its human ortholog. Numbers of amino acids are indicated; *numbers* in *parenthesis* indicate the size of the truncated protein product of 1a and 1c *ing1* isoforms. The *underlined methionine* in the mouse sequence indicates the beginning of the protein product encoded by isoforms 1a and 1c. The *asterisks* indicate identical amino acids, while *dots* indicate conserved changes in amino acid sequence. *B*, a multiple alignment of p33^{ING1}, its yeast homologs, and additional PHD finger-containing proteins. The aligned conserved blocks are separated by variable spacers whose lengths are indicated by *numbers*; for the N-terminal block, the distance to the protein N termini is indicated (the sequence of the human paralog of p33^{ING1} is incomplete). *Consensus1* shows amino acid conservation in the p33^{ING1} family of proteins; *Consensus2* shows the conservation in an expanded set of PHD finger proteins (in addition to the six proteins of the p33^{ING1} family, the sequences that aligned with p33^{ING1} with a probability of a random match below 10^{-4} in the first iteration of the PSI-BLAST analysis were included). Each consensus shows amino acid residues conserved in all sequences of the respective set; *h* indicates a hydrophobic residue, *p* indicates a polar residue, − indicates a negatively charged residue, *s* indicates a small residue, and *a* indicates an aromatic residue. The metal-chelating residues in the PHD finger domain are shown by *white type* on *black*. Each protein is identified by a gene name followed by species abbreviation and the gene identification number from the nonredundant protein data base at the NCBI. p33^{ING1}p is the human paralog of p33^{ING1}, Mi-2 is a human autoantigen, and CHD3 is a chromatin-associated helicase of the SNF2 family; the remaining proteins are uncharacterized gene products designated by their systematic gene names. Species abbreviations are as follows. *Hs*, *Homo sapiens*; *Sc*, *Saccharomyces cerevisiae*; *Sp*, *Schizosaccharomyces pombe*; *Ce*, *Caenorhabditis elegans*.

sion was found on the 11th day of development and was characterized by changes in the relative expression of different classes of mRNA transcripts.

Patterns of *ing1* expression in embryogenesis were also checked by *in situ* hybridization. We used the histoblotting technique, in which embryonic sections were fixed on a nitrocellulose membrane and then hybridized with the RNA probes corresponding to the common part of the *ing1* cDNA. Besides sense and antisense *ing1*-specific RNA probes, we also used an antisense probe for β-actin, a gene with ubiquitous expression, as an internal standard. Sections were prepared from 10-, 12-, 16-, and 18-day embryos. Results of *in situ* hybridization are shown in Fig. 5*C*. *ing1* is uniformly expressed in the whole mouse embryo at all stages of development examined. This is in agreement with the result obtained from the adult mouse tissues, where *ing1* is expressed ubiquitously and at similar levels in all organs analyzed. However, in day 10 embryos, higher expression levels were observed in the yolk sac, while at day 16 and 18 of development, higher levels of expression were detected in inner compartments of bones and probably match areas of ongoing ossification.

In order to determine patterns of expression of different *ing1* transcripts in mouse tissues and during embryogenesis, Northern blots were hybridized with probes corresponding to the alternative start sites of *ing1* (Fig. 5, *A* and *B*). Isoforms 1b and 1c were expressed ubiquitously in all adult mouse tissues

by *arrows* (the start site of isoform 1b was estimated based on the size of the 5′-RACE product and the analysis of genomic sequence). The initiator sequence of isoform 1a, including multiple initiation start sites, is *underlined*; TATA-like sequence for isoform 1b is shown in *boldface type*. The *underlined boldface* sequence is the CAAT box positioned about 100 base pairs upstream from the 1b transcript initiation site. Sp1 binding sites are displayed in *boldface italic type*. *B*, sequence of the putative promoter area of isoform 1c, with the *underlined* initiator sequence overlapping the two transcription start sites. Multiple Sp1 binding sites are also indicated. *C*, sequences of *ing1* cDNAs and their predicted protein products. Sequences of the alternative 1a, 1b, and 1c first exons as well as the common *ing1* exon are shown. The first ATG codon of isoform 1b is *underlined* as well as the ATG codon in the common exon that is used as the initiation codon for 1a and 1c translation. Stop codons, indicating the end of translation, are also *underlined*.

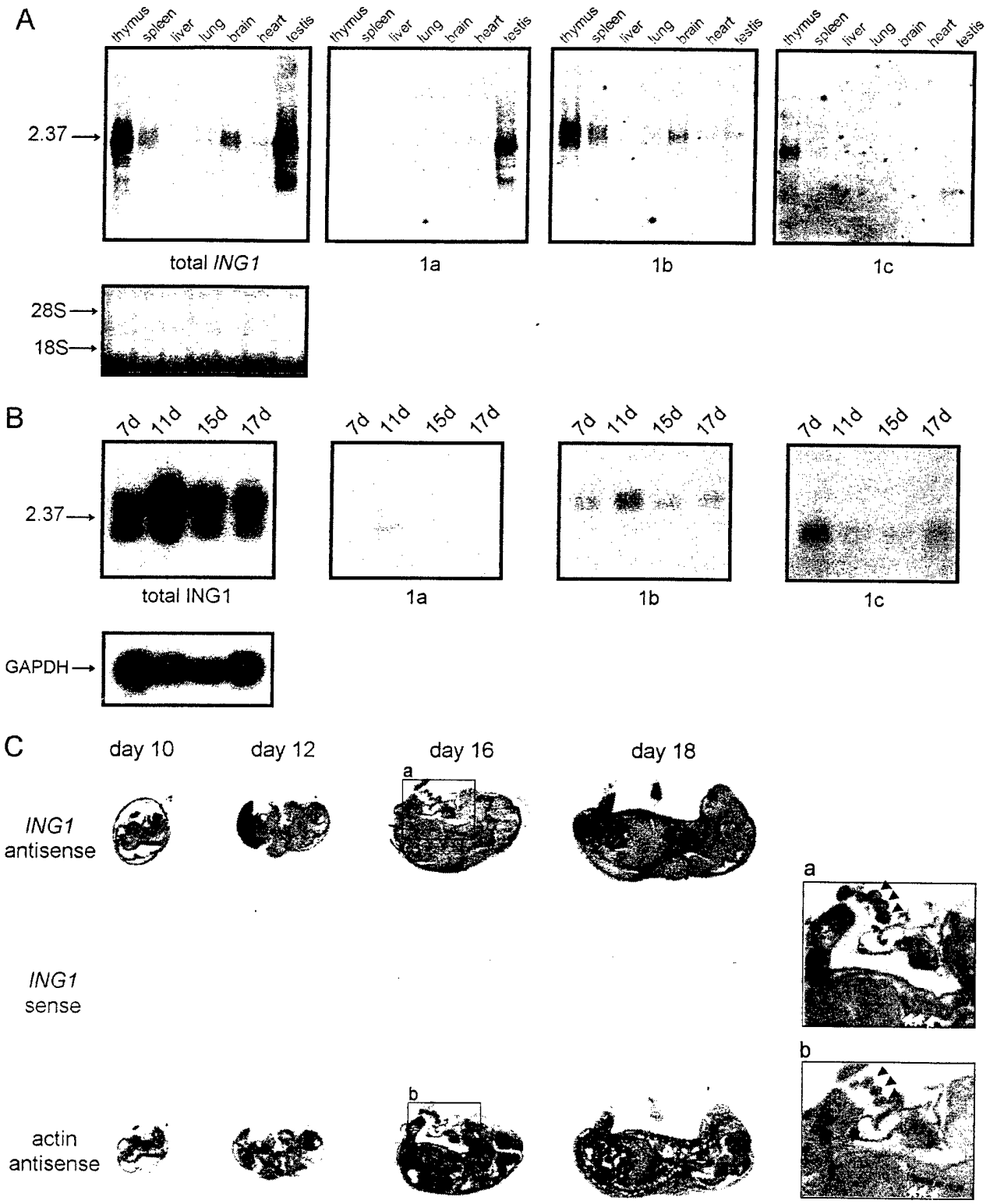


FIG. 5. ***ing1* expression in adult mouse tissues and embryos.** *A*, results of Northern hybridization of total mouse RNAs isolated from the indicated organs with the indicated probes; a photograph of an ethidium bromide-stained gel is shown as a loading control. *B*, Northern hybridization of RNA on a CLONTECH mouse embryonic multiple-tissue Northern blot (mRNA samples from mouse embryos at 7, 11, 15, and 17 days of development) with the same probes. The *arrows* indicate positions of the 2.37-kilobase RNA marker as well as the positions of 28 and 18 S rRNAs. *C*, *ing1* expression in mouse embryos analyzed by *in situ* histoblot hybridization. Histoblots were prepared from whole body sections of 10-, 12-, 16-, and 18-day embryos and hybridized with the sense and antisense *ing1*-specific ^{33}P-labeled RNA probes corresponding to the region encoding PHD domain. Antisense probe for ubiquitously expressed actin gene was used as a control.

tested, with the highest levels in thymus. Both isoforms were expressed in the embryos at all stages of development analyzed, with the highest levels at day 7 in the case of isoform 1c or day 11 in the case of isoform 1b. Out of all adult tissues analyzed, mRNA for isoform 1a was expressed only in testis and also in the 11-day embryo. While there is no detectable signal with the 1a-specific probe in the 7-day embryo, traces of hybridization could be detected in mRNA from day 15 and day

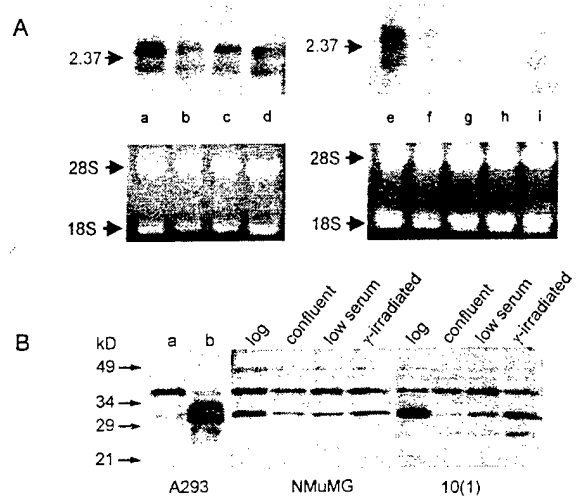


FIG. 6. ***ing1* mRNA and protein expression varies depending on cell growth conditions.** *A*, RNA was isolated from dividing 10(1) (*lane a*) and NMuMG (*lane e*) cell lines, as well as from the dividing young mouse embryonic fibroblasts (*lane h*). RNA was also isolated from contact-inhibited 10(1) (*lane b*) and NMuMG cells (*lane f*); serum-starved 10(1) cells (*lane c*) (64 h at 0.5% FBS); γ-irradiated 10(1) cells (*lane d*) and NMuMG cells (*lane g*); and senescent MEFs (*lane i*). Filters were probed with total *ing1* probe. The *bottom panels* show RNA gels from which Northern blots were made. The *arrows* indicate positions of the 2.37-kilobase RNA marker (*upper panels*) or 28 and 18 S rRNAs. *B*, detection of *ing1*-encoded proteins by Western immunoblotting, using antibodies against $p33^{ING1}$. Cell lysates were prepared using radioimmune precipitation buffer from the cells treated in the same way as explained above. As a control, cell lysates were prepared from 293 cells transfected with the plasmids expressing mouse 1b *ing1* variant (*lane a*) or the human homologue of $p31^{ing1}$ that produces the protein product of the same size as mouse 1c and 1a variants.

17 embryos, which indicates an extremely low expression level at these points of mouse embryogenesis.

Indications of Proliferation-dependent Regulation of ing1 Expression—To check whether *ing1* expression was proliferation-dependent, we analyzed RNA from two mouse cell lines, NMuMG and 10(1), at different growth conditions by Northern blot hybridization using total *ing1* cDNA as a probe. *ing1* expression was also compared in senescent *versus* young, dividing mouse embryonic fibroblasts. Results are shown in Fig. 6*A*. In both cell lines, *ing1* was expressed at higher levels in dividing compared with quiescent cells (quiescence is induced by serum starvation, contact inhibition, or γ-irradiation). However, in 10(1) cells the difference in expression was specific only for the upper *ing1* specific band, which corresponds to isoform 1b. In mouse embryonic fibroblasts, *ing1* was expressed at very low levels without any differences between dividing and senescent cells.

ing1 expression in cell lines was also analyzed by Western blotting using an IgG1 mouse monoclonal antibody produced against human recombinant $p33^{ING1}$ (16) (Fig. 6*B*). This antibody has previously been shown to specifically detect the denatured form of mouse $p33^{ING1}$ protein in Western immunoblot protocols. Cell lysates were produced from dividing, contact-inhibited, serum-starved, or γ-irradiated 10(1) and NMuMG cells. In both cell lines, two protein products were detected, 31 and 37 kDa in size, which correspond to the truncated *ING1* and 1b *ing1* protein products, respectively. The 37-kDa protein was present at the same levels in dividing and quiescent cells, while the 31-kDa protein was present at higher levels in dividing compared with nondividing cells. The variation of expression of *ing1* differs significantly at mRNA and protein levels, indicating that this gene could be regulated at the level of transcription or protein stability.

Overexpression of $p37^{ing1}$ Inhibits p53 Function—All previous functional analysis of *ING1* was done using human cDNA expressing the shorter protein encoded by this gene (1, 2, 4). As a result, $p33^{ING1}$ was defined as a negative growth regulator that cooperates with p53 in transcriptional activation of p53-responsive genes. Its mouse homologue, $p31^{ing1}$, has a similar biological effect (data not shown) that is consistent with the high degree of identity between the mouse and the human proteins (see Fig. 4). To analyze the influence of the longer product of *ing1* on p53 function, we overexpressed cDNA for $p37^{ing1}$ in mouse cells maintaining wild type p53. To effectively monitor p53-dependent transcriptional activation, we used previously characterized ConA cells (see "Materials and Methods"), carrying the p53-responsive bacterial *lacZ* gene. We transduced ConA cells with retrovirus expressing $p37^{ing1}$ and enhanced green fluorescent protein from a bicistronic mRNA and generated cell populations with different levels of overexpression of $p37^{ing1}$ by sorting cells differing in the levels of enhanced green fluorescent protein fluorescence. These cells were compared with ConA cells transduced with either the empty pLXIG vector or with retroviral vector expressing the short protein product of *ing1*. The expression of introduced constructs was confirmed by Western immunoblotting using an anti-$p33^{ING1}$ antibody that recognizes both human and mouse proteins (16) (Fig. 7*B*). p53-dependent expression of *lacZ* was determined by X-gal staining of ConA cells treated with UV light (Fig. 7*A*). The expression of p53 protein and p53-responsive $p21^{waf1}$ protein in untreated and in UV-irradiated cells was estimated by Western immunoblotting (Fig. 7*B*). A decreased intensity of the p21 band by Western blot and a weaker X-gal staining of ConA cells treated with UV light indicate that $p37^{ing1}$ clearly inhibited UV-induced accumulation of both p53-inducible proteins. Moreover, overexpression of $p37^{ing1}$ strongly attenuated accumulation of p53 in response to UV light (Fig. 7*B*). These observations demonstrate that $p37^{ing1}$ has a suppressive effect on p53-dependent transcriptional activation supposedly by attenuating p53 accumulation in response to DNA damage. This means that the two proteins encoded by the *ing1* gene have opposite effects on p53 function.

It was previously shown that $p33^{ING1}$ can be detected in cells in a complex with p53 by co-immunoprecipitation (4). Since at that time it was not known that *ING1* encodes two different proteins, it was unclear which of the two products of *ING1* was detected in these experiments. We therefore compared the ability of short and long *ING1*-encoded proteins to form a complex with p53 by using immunoprecipitation. Since the protocol of immunoprecipitation was optimized for human proteins, we analyzed human variants of *ING1* proteins expressed in human cells by transient transfection. p53-deficient Saos-2 cells were used in these experiments that have both alleles of the p53 gene deleted and presumably retain an intact p53 pathway. They were co-transfected in different combinations with the expression plasmids encoding wild type p53 and the short or long products of *ING1*. Cell lysates were treated with anti-p53 antibodies (see "Materials and Methods"), and the presence of *ING1*-encoded proteins in precipitates was detected by Western immunoblotting (Fig. 7*C*). The results obtained showed that although $p37^{ING1}$ is co-precipitated with p53, the shorter *ING1* product is not detectable in the p53 precipitate. This observation suggests that differences in the effect of the two *ING1* products on p53 function could be a reflection of differences in their interaction with p53 in the cell.

DISCUSSION

Multiple Products of the ing1 Gene—*ING1* was originally described as a gene whose suppression promotes neoplastic transformation (1). Consistent with that, ectopic expression of

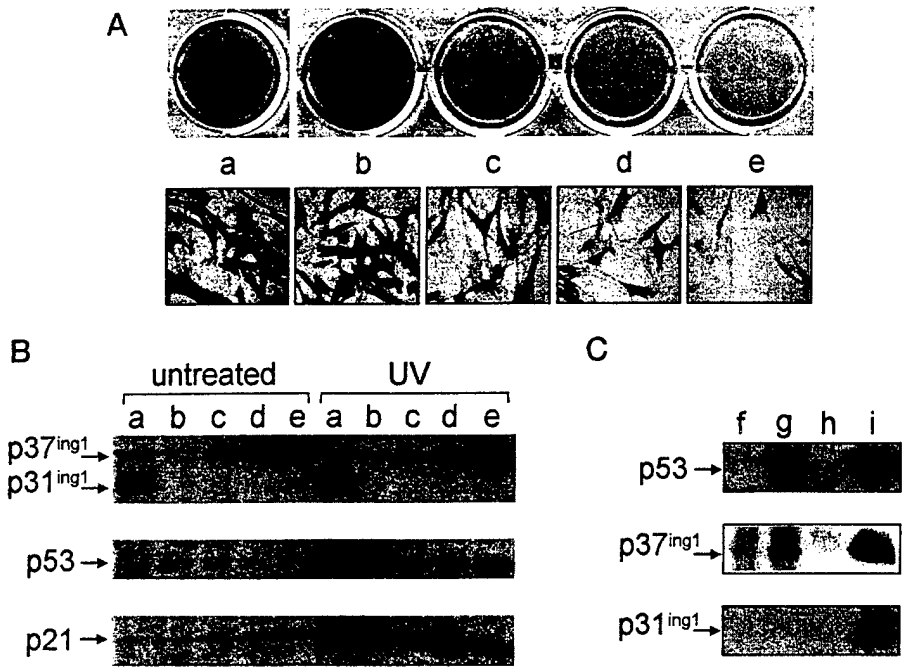


FIG. 7. **p37^{ing1} affects p53 function.** *A*, dependence of β-galactosidase activation after UV irradiation of ConA cells on the expression levels of p37^{ing1}. ConA cells expressing the human homologue of p31^{ing1} (*lane a*); p37^{ing1} at low (*lane c*), medium (*lane d*), or high (*lane e*) levels; and ConA cells infected with pLXIG vector alone (*lane b*) were equally plated in a 12-well plate, UV-irradiated (25 J/m^2), and X-gal-stained 16 h after the treatment. A fragment of the 12-well plate and microscopic views of individual wells are shown. *B*, expression of p53 protein, p53-responsive p21^{waf1} protein, and *ing1* protein products in untreated and in UV-irradiated ConA cells was estimated by Western immunoblotting. Analyzed cell lysates were isolated using radioimmune precipitation buffer from the cells treated in the same way as explained for *A*. *C*, coimmunoprecipitation of p53 and *ING1* protein products in Saos-2 cells. Detection of p53 (*upper panel*), p37^{ING1} (*middle panel*), and p31^{ING1} (*lower panel*) proteins by Western immunoblotting is shown. *Lanes f, g,* and *i* (*top* and *middle panels*), lysates from Saos-2 cells co-transfected with p53 and p37^{ING1}-expressing plasmids. *Lanes f, g,* and *i* (*bottom panel*), lysates from Saos-2 cells co-transfected with p53 and p31^{ING1}-expressing plasmids. *Lane h*, lysate from control nontransfected cells. *Lanes f* and *g* contain the products of immunoprecipitation with anti-p53 antibodies (*lane g*) and control nonspecific antibodies (*lane f*).

the first isolated human cDNA was growth-suppressive for different cell lines. *ING1* was found to encode a nuclear protein termed p33^{ING1} that was shown to functionally and physically interact with p53 in cell growth regulation (4, 22). Now, after we characterized the structure and expression of the mouse *ing1* gene, it became clear that *ing1* regulation is more complicated than was originally thought. *ing1* is transcribed from at least three differently regulated promoters, and the resulting transcripts encode at least two different proteins.

A similar type of regulation (alternative initiation leading to variability of 5′-exons) found in other tumor suppressor genes, namely *BRCA1*, *APC*, and *INK4* (23–26), is associated with the generation of proteins with different functions (in the case of *INK4*, different reading frames are translated in the alternative transcripts). Consistently, the two products of *ing1* have opposite effects on p53-dependent transcription regulation; one acts as a p53 cooperator, while the other acts as a p53 suppressor.

All of the transcripts share a common region encoded by a common exon but differ in their 5′-exons. Two of these alternative exons do not contain protein-coding sequences (isoforms 1a and 1c), while the third one does (isoform 1b). Consistently, one of the *ing1* transcripts encodes a 37-kDa protein (p37^{ING1}), while two others are translated into a shorter protein of 24 kDa that surprisingly runs as if it was 31 kDa (p31^{ING1}). Structures of mouse and human *ing1* genes are likely to be similar, considering the high degree of evolutionary conservation of *ing1* sequences and the fact that the two versions of human *ING1* that are currently available in GenBank™ also share a large common part but have different N termini. It is noteworthy that one of the human variants of *ING1* (GenBank™ accession number AF001954) does not have a homologue with similar 5′-sequences among the identified mouse *ing1* isoforms, which raises the possibility that there could be more variants of mouse *ing1* transcripts that so far have not been isolated.

p33^{ING1} and its homologs in such a distantly related species as yeast contain a remarkably conserved PHD finger domain and an additional, weakly conserved domain of unknown function. PHD fingers have been shown to bind DNA (21, 27), but to our knowledge there is no evidence that they mediate protein-protein interactions. Therefore, it seems likely that the C-terminal PHD domain in the p33^{ING} family of proteins is involved in specific DNA binding that may be important for transcription regulation. Given the outstanding conservation of this domain across the large phylogenetic distance that separates humans and yeast, the specificity of DNA binding with respect to particular binding sites is expected to be conserved either in terms of DNA sequence, distinct features of chromatin structure, or both. It may be further surmised that the conserved N-terminal domain is involved in specific protein-protein interactions that couple transcription regulation by p33^{ING1} and its homologs to other elements of cell cycle control. The conservation of domain organization in p33^{ING1} and its yeast homologs is particularly notable given that yeast does not encode any homologs of p53. Thus, it appears that p33^{ING1} is an ancient cell cycle regulator whose interaction with p53 is a later evolutionary addition.

Regulation of ing1 Expression—The results of expression analysis indicated that mouse *ing1* is a subject of regulation both at the protein and mRNA levels and that different isoforms have different expression patterns. Thus, p37^{ING1} is ubiquitously expressed in all tissues analyzed with elevated mRNA expression levels in the thymus, while levels of the p31^{ING1} protein vary dramatically among organs and cell lines.

Sizes of the *ing1* mRNA transcripts detected by Northern hybridization correlate well with the length of cDNA sequences isolated from all of the tissues except testis. Probes specific for each of the identified splice variants reveal multiple RNA bands, indicating a variability of the *ing1* transcripts in this organ. It is likely that in testis *ing1* mRNAs are either terminated or processed differently from the rest of the tissues.

There is a clear link between the proliferation rate of the cell and *ing1* expression both at the protein and mRNA levels. This observation may reflect cell cycle dependence of *ing1* transcription detected earlier for the human *ING1* gene; the biological significance of this regulation is not obvious so far. We failed to observe up-regulation of mouse *ing1* in senescent cells as it has been reported for human *ING1* (8), which suggests that regulation patterns of mouse and human *ing1* genes may not always be the same.

ING1 and p53: Cooperators or Antagonists?—Human *ING1* has been known as a cooperator of p53 involved in negative growth regulation (1, 4), apoptosis (3), and senescence (2). This set of properties, together with the observations that show a decreased expression of *ING1* in several breast carcinoma cell lines and *ING1* gene rearrangement in one neuroblastoma cell line, allow ING1 to be defined as a candidate tumor suppressor (1–4, 16, 22). Here we demonstrate that *ING1* encodes, in addition to the protein with the above described properties, another product that shows an opposite effect on p53 function, inhibiting p53-dependent transcriptional activation. Thus, a single *ING1* gene appeared to simultaneously encode a candidate tumor suppressor (p53 cooperator) and a putative oncogene (p53 inhibitor). This means that a simplified view of the potential role of *ING1* in cancer should be revised. In the case of *ING1*, we may have a new genetic mechanism of promoting cancer that involves an imbalance between the two products of one gene. Further characterization of the biological activity of the long product of *ING1*, $p37^{ING1}$, indicated that it can affect the whole spectrum of cell properties known to be controlled by p53, including sensitivity to apoptosis, replicative senescence, cooperation with dominant oncogenes, and drug response.[2] Thus, overexpression of the longer product of *ING1*, which functionally acts as a p53 suppressor, could be the mechanism of attenuation of p53 activity in tumors that do not require mutations in p53 itself.

A large scale analysis of clinical samples is needed to conclude whether this hypothesis is correct or not. However, any attempts to investigate the potential cancer relevance of *ING1* should involve separate analysis of the alternative isoforms of this gene. It has already become obvious that a direct measurement of the overall mRNA expression in tumors cannot be used as an approach to judge the involvement of *ING1* in carcinogenesis. The ratio between expression levels of the two *ING1* products seems to provide much more meaningful information. It is therefore essential to establish such assays that would distinguish between the two protein products or among the alternative *ING1* transcripts.

Although the mechanism of p53 modulation by the two products of the *ING1* gene remains unknown, we hope that the two new observations described in this work will shed light on this problem. We found that (i) p53 is found in a complex with the long but not with the short product of *ING1* and (ii) overexpression of the longer product inhibits accumulation of p53 protein after DNA damage. Both properties of $p37^{ING1}$ clearly resemble those of the natural p53 antagonist, Mdm2, which was shown to affect nuclear localization of p53 (28, 29) and to promote its proteasomal degradation (30, 31). All of these possibilities should be tested for $p37^{ING1}$ as well as a potential interdependence of the biological activity of these two proteins.

Is the function of *ING1* limited to its interaction with the p53 signaling pathway? It seems doubtful, considering the high evolutionary conservation of *ING1*. In fact, *ING1* homologues found in yeast (Fig. 4), which does not have detectable *p53*-related genes, suggest some important p53-independent role for *ING1*. The phenotypes associated with the knockout of *ING1* in yeast and mice may provide a lead toward its currently unknown cellular function. It is noteworthy that a gene knockout approach will be complicated by the necessity to separately inactivate expression of each of the two products of *ING1*.

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REFERENCES

1. Garkavtsev, I., Kazarov, A., Gudkov, A., and Riabowol, K. (1996) *Nat. Genet.* **14,** 415–420
2. Garkavtsev, I., and Riabowol, K. (1997) *Mol. Cell. Biol.* **17,** 2014–2019
3. Helbing, C. C., Veillette, C., Riabowol, K., Johnston, R. N., and Garkavtsev, I. (1997) *Cancer Res.* **57,** 1255–1258
4. Garkavtsev, I., Grigorian, I. A., Ossovskaya, V. S., Chernov, M. V., Chumakov, P. M., and Gudkov, A. V. (1998) *Nature* **391,** 295–298
5. Miller, A. D., and Rosman, G. J. (1989) *BioTechniques* **7,** 980–986
6. Jang, S. K., Krausslich, H. G., Nicklin, M. J., Duke, G. M., Palmenberg, A. C., and Wimmer, E. (1988) *J. Virol.* **62,** 2636–2643
7. Cormack, B. P., Valdivia, R. H., and Falkow, S. (1996) *Gene (Amst.)* **173,** 33–38
8. Harvey, D. M., and Levine, A. J. (1991) *Gen. Dev.* **5,** 2375–2385
9. Pear, W. S., Nolan, G. P., Scott, M. L., and Baltimore, D. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 8392–8396
10. Komarova, E. A., Chernov, M. V., Franks, R., Wang, K., Armin, G., Zelnick, C. R., Chin, D. M., Bacus, S. S., Stark, G. R., and Gudkov, A. V. (1997) *EMBO J.* **16,** 1391–1400
11. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
12. Chenchik, A., Zhu, Y. Y., Diatchenko, L., Li, R., Hill, J., and Siebert, P. D. (1998) *Gene Cloning and Analysis by RT-PCR* (Siebert, P. D., and Larrick, J. W., eds) pp. 305–319, BioTechniques Books, ——, ——
13. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) *Nucleic Acids Res.* **25,** 3389–3402
14. Neuwald, A. F., Liu, J. S., and Lawrence, C. E. (1995) *Protein Sci.* **8,** 1618–1632
15. Schuler, G. D., Altschul, S. F., and Lipman, D. J. (1991) *Proteins* **9,** 180–190
16. Garkavtsev, I., Boland, D., Mai, J., Wilson, H., Veillette, C., and Riabowol, K. (1997) *Hybridoma* **16,** 537–540
17. Gudkov, A. V., Kashkin, K. N., Zaitsevskaya, T. E., and Troyanovsky, S. M. (1989) *Int. J. Cancer* **44,** 1052–1056
18. Smale, S. T. (1994) *Transcription: Mechanisms and Regulation* (Conaway, R. C., and Conaway, J. W., eds) pp. 63–80, Raven Press, New York
19. Smale, S. T. (1997) *Biochim. Biophys. Acta* **1351,** 73–88
20. Aasland, R., Gibson, T. J., and Stewart, A. F. (1995) *Trends Biochem. Sci.* **20,** 56–59
21. Schindler, U., Beckmann, H., and Cashmore, A. R. (1993) *Plant J.* **4,** 137–150
22. Garkavtsev, I., Demetrick, D., and Riabowol, K. (1997) *Cytogenet. Cell Genet.* **76,** 176–178
23. Cui, J. Q., Wang, H., Reddy, E. S., and Rao, V. N. (1998) *Oncol. Rep.* **5,** 585–589
24. Quelle, D. E., Zindy, F., Ashmun, R. A., and Sherr, C. J. (1995) *Cell* **83,** 993–1000
25. Thliveris, A., Samowitz, W., Matsunami, N., Groden, J., and White, R. (1994) *Cancer Res.* **54,** 2991–2995
26. Xu, C.-F., Brown, M. A., Chambers, J. A., Griffiths, B., Nicolai, H., and Solomon, E. (1995) *Hum. Mol. Genet.* **4,** 2259–2264
27. Stassen, M. J., Bailey, D., Nelson, S., Chinwalla, V., and Harte, P. J. (1995) *Mech Dev.* **52,** 209–223
28. Freedman D. A., and Levine, A. J. (1998) *Mol. Cell. Biol.* **18,** 7288–7293
29. Roth, J., Dobbelstein, M., Freedman, D. A., Shenk, T., and Levine, A. J. (1998) *EMBO J.* **17,** 554–564
30. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997) *Nature* **387,** 296–299
31. Kubbutat, M. H., Jones, S. N., and Vousden, K. H. (1997) *Nature* **387,** 299–303

[2] I. A. Grigorian, K. V. Gurova, M. Zeremski, S. S. Kwek, and A. V. Gudkov, manuscript in preparation.